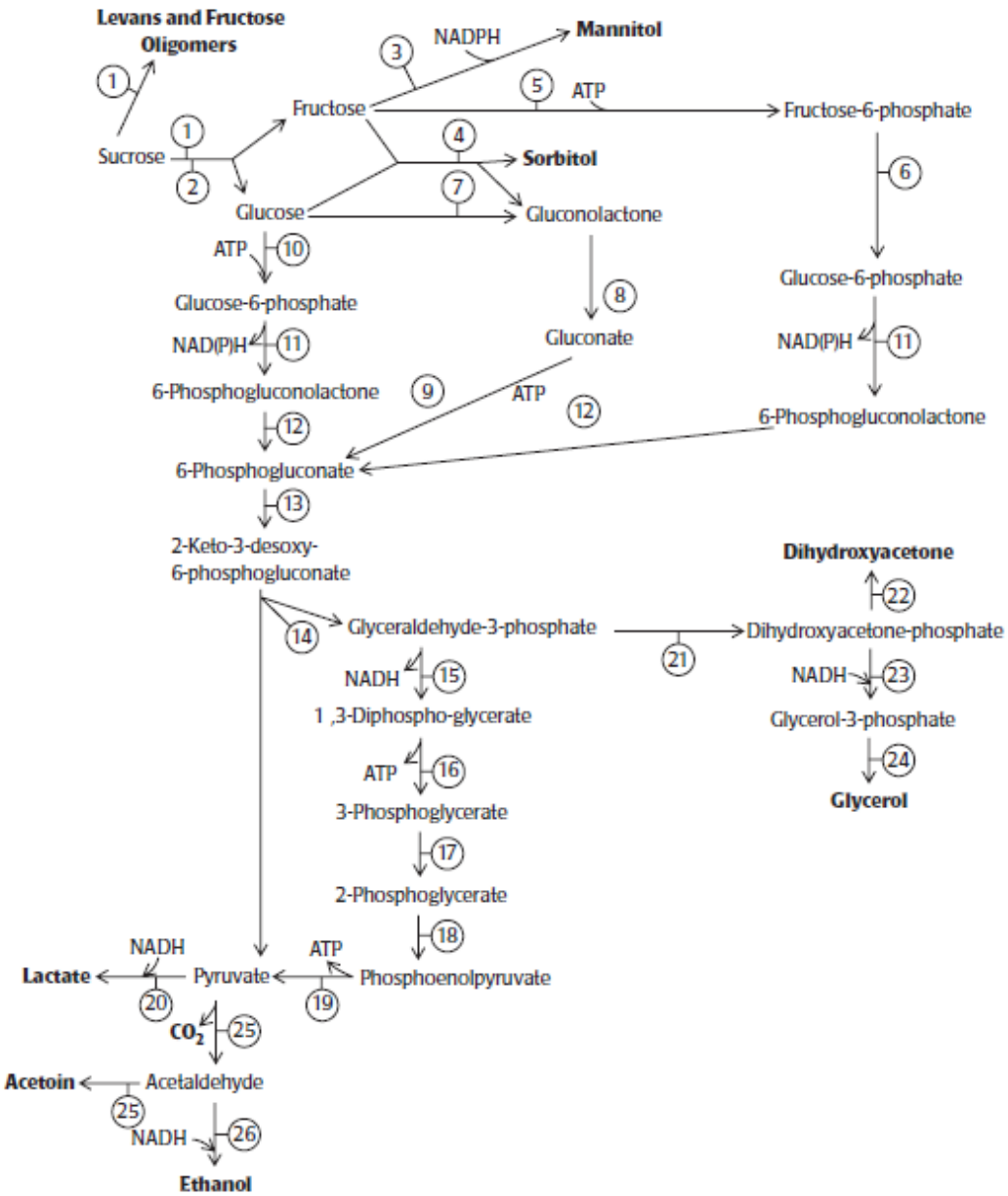
Unit-III Fermentation Technology: (10 Lectures)

Industrial production of chemicals: Ethanol, Acids (citric, acetic and gluconic acid), Solvents (glycerol, acetone, butanol), Antibiotics (penicillin, streptomycin, tetracyclin), Semi-synthetic antibiotics, Amino acids (lysine, glutamic acid), Single cell protein.

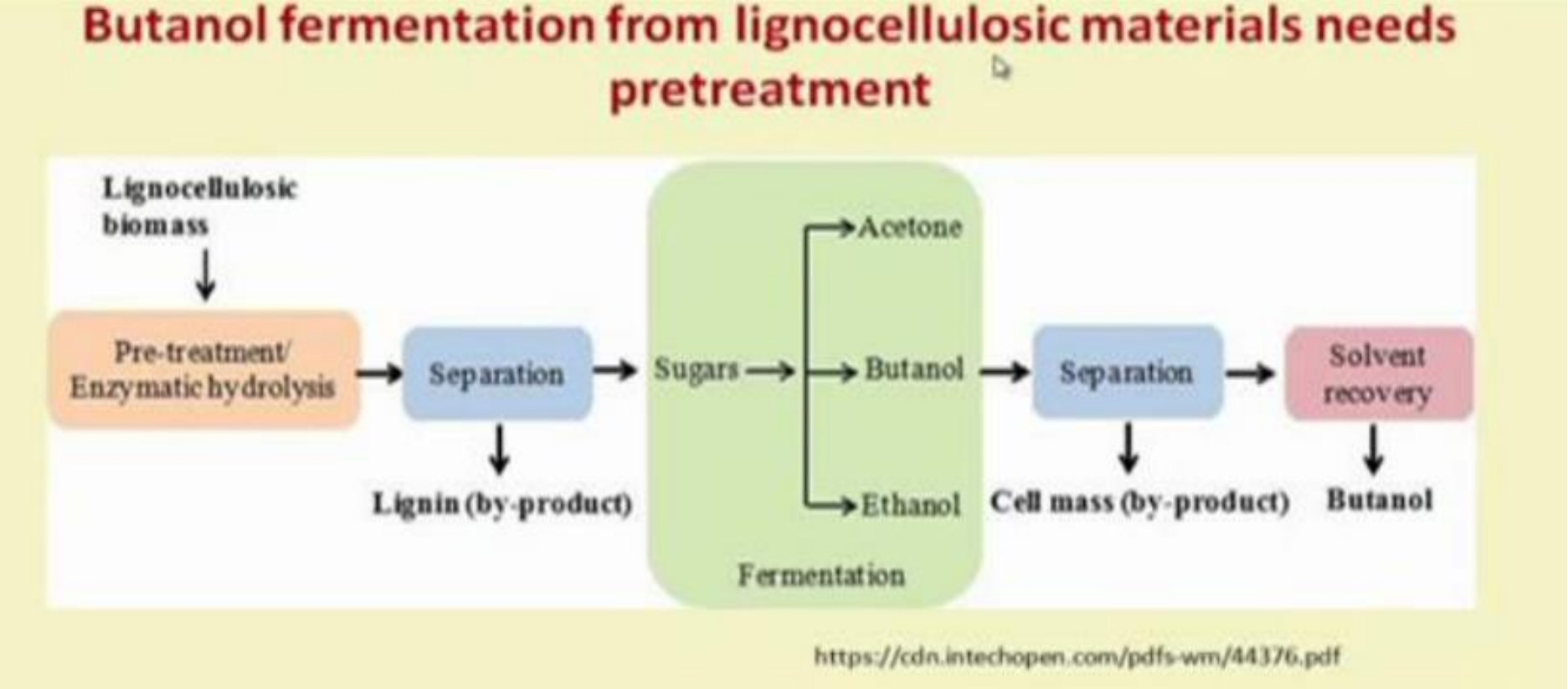
TABLE 13.1 Some yeasts and bacteria that produce significant quantities of ethanol, and the major carbohydrates utilized as substrates

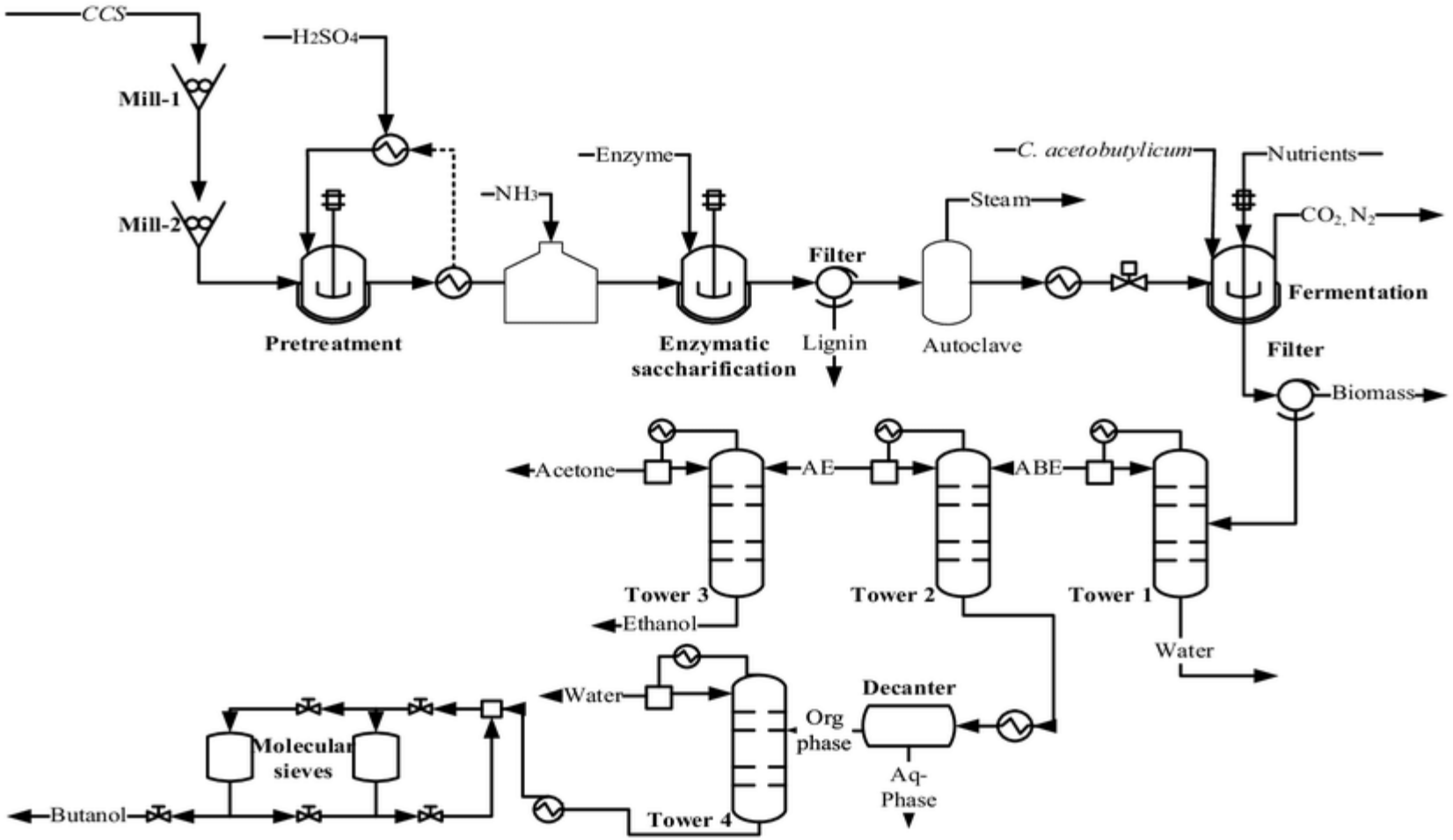
Yeast or Bacteria	Substrates
Yeast	
Saccharomyces spp.	
S. cerevisiae	Glucose, fructose, galactose, maltose, maltotriose, xylulose
S. carlsbergensis	Glucose, fructose, galactose, maltose, maltotriose, xylulose
S. rouxii (osmophilic)	Glucose, fructose, maltose, sucrose
Kluyveromyces spp.	
K. fragilis	Glucose, galactose, lactose
K. lactis	Glucose, galactose, lactose
Candida spp.	
C. pseudotropicalis	Glucose, galactose, lactose
C. tropicalis	Glucose, xylose, xylulose
Bacteria	
Zymomonas mobilis	Glucose, fructose, sucrose
Clostridium spp.	
C. thermocellum (thermophilic)	Glucose, cellobiose, cellulose
C. thermohydrosulfuricum (thermophilic)	Glucose, xylose, sucrose, cellobiose, starch
Thermoanaerobium brockii (thermophilic)	Glucose, sucrose, maltose, lactose, cellobiose, starch
Thermobacterioides acetoethylicus (thermophilic)	Glucose, sucrose, cellobiose

Metabolism of sucrose, glucose and fructose in *Zymomonas mobilis*. Enzymes are as follows: 1, levansucrase; 2, invertase; 3, mannitol dehydrogenase; 4, glucose-fructose oxidoreductase; 5, fructokinase; 6, glucose-6-phosphate isomerase; 7, glucose dehydrogenase; 8, gluconolactonase; 9, gluconate kinase; 10, glucokinase; 11, glucose-6-phosphate dehydrogenase; 12, 6-phosphogluconolactonase; 13, 6-phosphogluconate dehydratase; 14, keto-deoxy-phosphogluconate aldolase; 15, glyceraldehyde 3-phosphate dehydrogenase; 16, phosphoglycerate kinase; 17, phosphoglycerate mutase; 18, enolase; 19, pyruvate kinase; 20, lactate dehydrogenase; 21, triose-phosphate isomerase; 22, phosphatase; 23, glycerol 3-phosphate dehydrogenase; 24, phosphatase; 25, pyruvate decarboxylase; 26, alcohol dehydrogenase.



Clostridia are natural acetone-butanol-ethanol (ABE) fermentation organisms. They are spore forming bacteria




CCS
Mill-1
Mill-2
H2SO4
Pretreatment
NH3
Enzyme
Enzymatic saccharification
Filter
Lignin
Steam
Autoclave
C. acetobutylicum
Nutrients
CO2, N2
Fermentation
Filter
Biomass
Acetone
AE
ABE
Tower 3
Tower 2
Tower 1
Ethanol
Water
Water
Decanter
Org phase
Aq-Phase
Tower 4
Molecular sieves
Butanol

Stillage

Stillage is the residue from the first distillation of fermented substrate (corn mash, sugarcane juice). With sugarcane, about 12 L of stillage are produced for each liter of ethanol. Such stillage contains 40 to 65 g of organic matter per liter. Depending on what is done with it, stillage is either a serious water-polluting waste or a source of valuable byproducts.

TABLE 13.2 Major components of stillage remaining after alcohol distillation from fermented sugar cane juice

Component	Amount remaining (g/L)
Organic matter	40–65
Nitrogen	0.7–1.0
Phosphorus	0.1–0.2
Potassium	4.5–8.0

GLYCEROL AS BYPRODUCT DURING BIODIESEL PRODUCTION

- Glycerol (also known as glycerin) is a major byproduct in the biodiesel manufacturing process.
- Biodiesel production will generate about 10% (w/w) glycerol as the main byproduct.
- In general, for every 100 pounds of biodiesel produced, approximately 10 pounds of crude glycerol are created.
- It is projected that the world biodiesel market would reach 37 billion gallons by 2016, which implied that approximately 4 billion gallons of crude glycerol would be produced

- Biodiesel production produce ~10 % w/w of crude glycerol.
- Increase in biodiesel production ,Increase in crude glycerol.
- Surplus crude glycerol,Decrease in price of pure glycerol
- Conversion of crude glycerol into value added products is essential to maintain the sustainability of biodiesel production.

Composition of crude glycerol.

- Glycerol
- Light solvent (Methanol or ethanol, water)
- Soap
- Fatty acid methyl esters (FAME i.e. biodiesel
- Glycerides (i.e. to mono, di & tri-glycerides),
- Free fatty acids (FFA)
- Ash

FACTORS AFFECTING THE COMPOSITION OF CRUDE GLYCEROL

- Catalyst type used- lipase, alkaline,acidic
- Transesterification efficiency
- Recovery efficiency of biodiesel
- Impurities in feedstock
- Recovery efficiency of methanol and catalyst

Excess methanol is used during transesterification to enhance the yield

Excess methanol distributes between the methyl-ester and crude glycerol phase.

However, due to toxicity of methanol on environment & health it has to be removed and recycled back CG can be treated under vacuum conditions using a rotary evaporator at 50-90 0C for more than 2 h

In industry, commonly evaporator or flash unit is employed Falling film evaporators recommended as best

As it keeps shorter contact time

Prevents glycerol decomposition Glycerol concentration after methanol removal= ~85% 1

NEUTRALIZATION

- Most common pretreatment method involves acidifying with Strong acid to remove Catalyst and Soaps Acid + Soap
- Free fatty acids (skimmed off) Acid + Base catalyst (NaOH, KOH) Salt + Water Acidification process usually separates the crude glycerol into 3 layers:
- 1. free fatty acids Top layer (Separated using separated funnel)
- 2. glycerol rich layer Middle &
- 3. Inorganic saltsBottom

PURIFICATION AND REFINERY

Vacuum

↓

Distillation

↓

Ion Exchange Adsorption

↓

Adsorption using activated carbon

- At higher temperature glycerol property changes,
- >200 0C, Polymerization into polyglycerol
- >160 0C & slightly acidic condition, dehydration

- Can oxidize into glycerose, glyceraldehydes and di-hydroxylacetone

- Thus, purification has to be done in vacuum where the pH, temperature and pressure must be controlled

USES OF GLYCEROL

- Large quantity of glycerine is consumed in the manufacture of nitro-glycerine, cosmetics and medicinal preparations.
- It is also used in the production of printer's roller and of inks for use in rubber stamps.
- The hydroscopic properties of glycerol cause it to be used in keeping tobacco moist and to keep leather soft.
- Glycerol is used in the preparation of personal care products, such as skin, hair and soap products (23 percent) and in oral hygiene products, such as toothpastes and mouthwashes (17 percent).

Butanol fermentation

Sucrose and starch

Butanol has traditionally been produced by ABE fermentation using carbon substrates obtained from corn, sugar beets and sugar cane, potatoes, tapioca and millet. As *Clostridium* sp. possess strong amylase activities, they can effectively utilize starchy substrates without the need for hydrolysis. Molasses, whey permeate, cassava and Jerusalem artichokes have also been used as fermentation substrates for butanol production.

Algae as an alternative substrate

Biofuel production using terrestrial biomass will only be beneficial if the biomass can be produced in a sustainable way without affecting environment and biodiversity; if not, the food *vs*. fuel debate surges again. Fuels based on terrestrial plants are controversial because they require cultivation area that could otherwise be used for growing food crops intended to supply food, feed and fiber to an expanding world population. A possible solution for this is the utilization of abundantly

Advances in fermentation process development for improved butanol production

In order to establish a highly productive and cost competitive butanol fermentation system, attempts have been made to develop new fermentation strategies using inexpensive carbon substrates. Efforts to reduce the cost of fermentation substrates by utilization of alternative substrates have been reviewed in the previous section. In the present section, some of the innovative strategies recently developed to overcome the problems in butanol production by ABE fermentation are described

Table 2. Recent fermentation and recovery process development for improved butanol production (since 2009).

Fermentation and recovery process	Microorganism	Strain	Strategy	Performance	Reference
Batch	*C. butylicum*	TISTR 1032	Co-culture of amylase producing *Bacillus subtilis* WD 161 with solventogenic clostridia	10-fold increase in amylase activity; butanol production from starch was improved by 5.4–6.5-fold	(Tran et al., 2010)
Fed-batch	*C. saccharoper-butylacetonicum*	N14	pH stat continuous lactic acid and glucose feeding	Production of 15.5 g/l butanol with 1.76 g/l/h productivity	(Oshiro et al., 2010)
Continuous	*C. pasteurianum*	MBEL_GLY2	High cell recycling using glycerol	710 h fermentation without strain degeneration, 7.8 g/l/h of butanol productivity	(Malaviya et al., 2012)
In situ product recovery	*C. acetobutylicum*	ATCC 824	*In situ* recovery of butanol by adsorption on polymeric resins	By application of 0.05 kg/L DowexOptipore SD-2 resin, 22.2 g/l butanol was obtained	(Nielsen and Prather, 2009)
	C. acetobutylicum	ATCC 824	Pervaporation processby application of a new polydimethylsiloxane (PDMS)/dual support compositemembrane	Max. volumetric glucose consumption rate increased 3.15 from 2.64 g/L/h (no pervaporation condition); average volumetric glucose consumption rate increased up to 2.11 from 1.62 g/L/h	(Li et al., 2011)

		compositemembrane	pervaporation condition); average volumetric glucose consumption rate increased up to 2.11 from 1.62 g/L/h	
C. acetobutylicum	JB200	Fibrous bed bioreactor with gas stripping	76.4 g/l butanol with a yield of 0.23 g/g substrate and a volumetric productivity of 0.29 g/l/h	(Lu et al., 2012)
C. acetobutylicum	JB200	Gas stripping applied in fed-batch fermentation	Production of 113.3 g/L butanol from 474.9 g/L glucose during 326 h	(Xue et al., 2012)
C. acetobutylicum	PJC4BK (pIPA3-Cm2)	Gas stripping applied in fed-batch fermentation	Production of 26 g/L butanol (35.6 g/L of IBE) during 45 h	(Lee et al., 2012a)
C. beijerinckii	P260	Vacuum process (continuous, 4-h, 6-h, 8-h interval vacuum) with 7-L fermentation volume	76.4 g butanol production with productivity of 0.23 g/l/h (continuous); 85.1 g butanol production with productivity of 0.27 g/l/h (4-h interval); 103.0 g butanol production with productivity of 0.23 g/l/h (6-h interval); 84.6 g butanol production with productivity of 0.19 g/l/h (8-h interval)	(Mariano et al., 2011)

Fermentation coupled with *in situ* product recovery

Butanol toxicity is one of the most critical problems of ABE fermentation. This limits the concentration of carbon substrate that can be used during fermentation and eventually results in low butanol concentration and productivity. In order to overcome the problem of butanol toxicity, *in situ* product recovery (ISPR) techniques have been developed with an aim to improve the process performance by reducing the product toxicity. As reviewed before, several ISPR techniques including adsorption, liquid–liquid extraction, perstraction (a combined membrane permeation and extraction by contacting the fermentation broth with an extracting solvent), reverse osmosis, pervaporation, and gas stripping have been evaluated for their efficiency and performance with respect to butanol production and recovery during ABE fermentation.

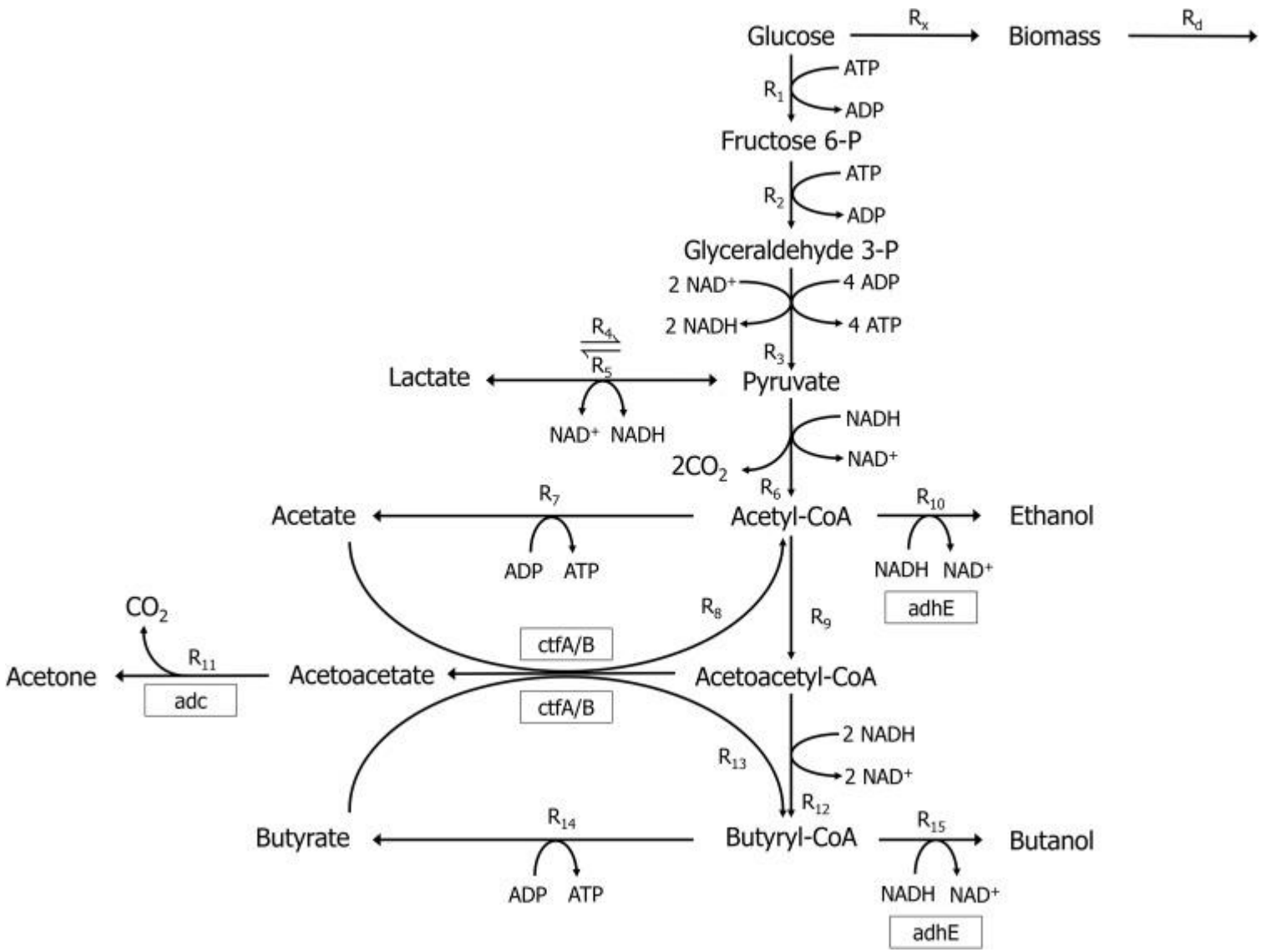
- Pervaporation
- *In situ* product recovery by adsorption
- Gas stripping
- Vacuum-based *in situ* product recovery

Acetone Production

Acetone is produced by fermentation through the acetone-butanol-ethanol (ABE) process, also known as the Weizmann process

Biobutanol can be produced by bacteria of genus *Clostridium* (*C*.) in a process known as the acetone-butanol-ethanol (ABE) fermentation.

The ABE fermentation is a biphasic process that converts sugars into acids (acetate, butyrate) and solvents (acetone, butanol, ethanol). During the first phase, acidogenesis, the primary products are the acidic metabolites. As the metabolism shifts to solventogenesis, the acids are assimilated into the ABE solvents. While this metabolic shift is associated with changes in the extracellular pH and the onset of sporulation, its exact mechanism is not understood. Recent experiments have shown that enzyme regulation plays a key role in the phase shift. The ABE fermentation is also dependent on various culture conditions such as pH, nutrient shortage, product inhibition, media composition, and redox state. The most prevalent Clostridium bacteria for the ABE fermentation are *C. acetobutylicum* and *C. beijerinckii*, though the more recent *C. saccharoperbutylacetonicum* N1-4 strain has garnered interest due to high butanol yields.


Glucose
R_x
Biomass
R_d
R_1
ATP
ADP
Fructose 6-P
R_2
ATP
ADP
Glyceraldehyde 3-P
2 NAD+
4 ADP
2 NADH
4 ATP
R_3
R_4
R_5
Lactate
Pyruvate
NAD+ NADH
NADH
NAD+
$2CO_2$
R_6
R_7
Acetate
Acetyl-CoA
R_{10}
Ethanol
ADP ATP
NADH NAD+
adhE
R_8
R_9
CO_2
ctfA/B
R_{11}
Acetone
Acetoacetate
Acetoacetyl-CoA
adc
ctfA/B
2 NADH
2 NAD+
R_{13}
R_{12}
R_{14}
Butyrate
Butyryl-CoA
R_{15}
Butanol
ADP ATP
NADH NAD+
adhE

In order to enhance the ABE fermentation yield, mainly in situ product recovery systems have been developed. These include gas stripping, pervaporation, liquid–liquid extraction, distillation via Dividing Wall Column, membrane distillation, membrane separation, adsorption, and reverse osmosis.

Production of Amino Acids by the Direct Fermentation

The production of amino acids by fermentation was stimulated by the discovery of an efficient L- glutamic acid producer *Corynebacterium glutamincum.* Many microorganisms have been reported to produce amino acids. They are mainly bacteria, but they also include some molds and yeasts. The four most widely reported bacteria belong to the following four genera. *Corynebacterium* spp. (*C. glutamicum; C. lilum*) *Brevibacterium* spp. (*B. divericartum: B. alanicum*) *Microbacterium* spp. (*M. flavum* var. *glutamicum*) *Arthrobacter* spp. (*A. globiformis; A. aminofaciens*) Auxotrophic and regulatory mutants of glutamic acid producing bacteria are used for the commercial production of all amino acids outside L- glutamic acid and L-glutamine, which are produced by the wild type of these organisms

Wild-type		*Auxotrophic Mutants*		*Regulatory Mutants*	
L- glutamic acid	1	L- citruline	1	L- arginine	1,2,3
L-valine,	1	L- leucine	1	L- histidine	1,3,5
		L- lysine	1	L- isoleucine	1,3,5
		L- ornithine	1	L- leucine	5,6
		L- proline	3	L- lysine	3
		L- threonine	4	L- methionine	1
		L- tyrosine	1	L- phenylalanine	1,3
				L- thereonine	1,3
				L- tryptophane	1,3
				L- tyrosine	1,3
				L- valine	6

1 = *Corynebacterium glutamicum*
2 = *Bacillus subtilis*
3 = *Brevibacterium flavum*
4 = *Escherichia coli*
5 = *Serratia marcescens*
6 = *Brevibacterium lactofermentum*

PRODUCTION OF GLUTAMIC ACID BY WILD TYPE BACTERIA

(i) *Organisms*: Wild type strains of the organisms of the four genera mentioned above are now used for the production of glutamic acid. The preferred organism is *Corynebacterium glutamicum.*

The properties common to the glutamic acid bacteria are: (a) they are all Gram-positive and non-motile; (b) they require biotin to grow; (c) they lack orhave very low amounts of the enzyme –ketoglutarate dehydrogenase, which is formed by removal of CO2 from isocitrate formed in TCA cycle (citric acid cylce). Since -ketoglutarate is not dehydrogenated it is available to form glutarate by reacting with ammonia.

(ii) *Conditions of the fermentation*: The composition of a medium which has been used for the production of glutamic acid is as follows (%): glucose, 10; corn steep liquor 0.25; enzymatic casein hydrolysate 0.25; K2HPO4 0.1, Mg. SO4, 7H2 O, 0.25; urea, 0.5. It should be noted that besides glucose, hydrocarbons have served as carbon sources for glutamic acid production. The optimal temperature is 30° to 35° and a high degree of aeration is necessary.

(iii) *Biochemical basis for glutamic acid production*: Studies by several workers have clarified the basis for glutamic acid production as summarized below.

(a) Glutamic acid production is greatest when biotin is limiting; that is, when it is suboptimal. When biotin is optimal, growth is luxuriant and lactic acid, not glutamic acid, is excreted. The optimal level of biotin is 0.5 mg per gm of dry cells; with higher amounts glutamic acid production falls.

(b) The isocitrate-succinate part of the TCA cycle is needed for growth. It is only after the growth phase that glutamic acid production becomes optimal.

(c) An increase in the permeability of the cell is necessary so as to permit the outward diffusion of glutamic acid, essential for high glutamic acid productivity.

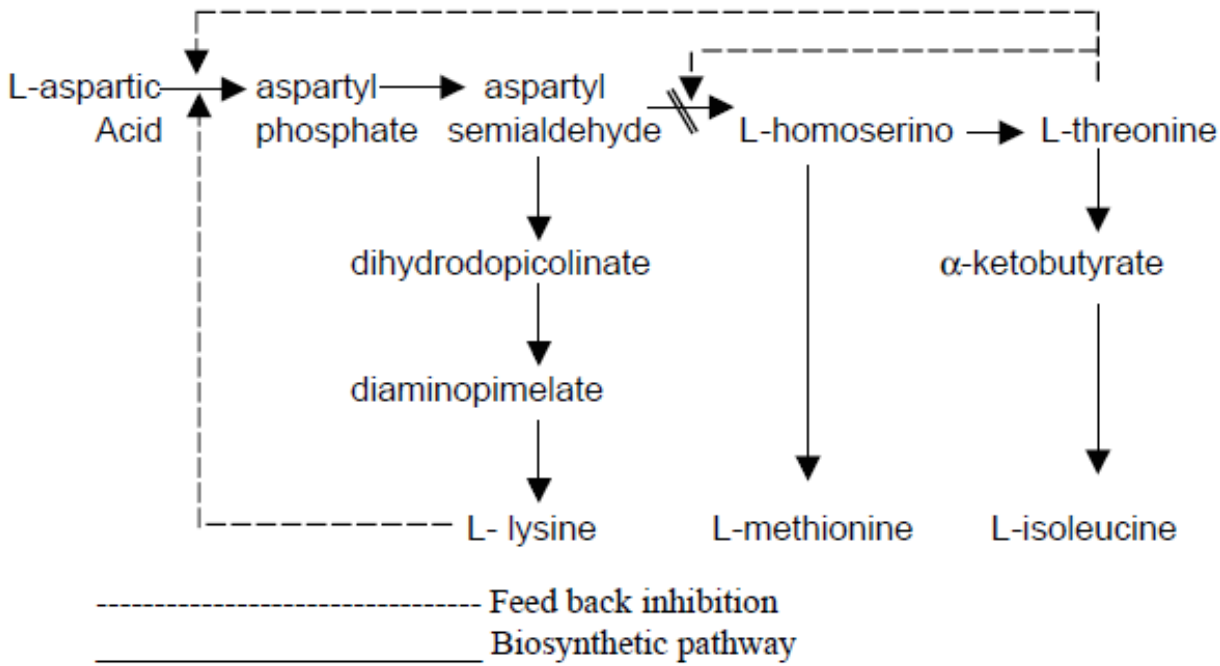
This increased permeability to the acid can be achieved in the following ways: (i) ensuring biotin deficiency in the medium (ii) treatment with fatty acid derivatives, (iii) ensuring oleic acid deficiency in mutants requiring oleic acid (C16 - C18**). (iv) addition of penicillin during growth of glutamic acid bacteria,** Cells treated in one of the first three ways above have cell membranes in which the saturated to unsaturated fatty acid ratio is abnormal, therefore the permeability barrier is destroyed and glutamic acid accumulates in the medium. The major factor in glutamic acid production by wild type organism is thus altered permeability.

Treatment with penicillin prevents cell-wall formation.

Cell wall inhibiting antibiotics such as penicillin and cephalosporin have enabled the use of molasses which are rich in biotin for glutamic acid production.

Production of Amino Acids by Auxotrophic Mutants

The first to be produced was L- lysine using limiting concentrations of either L- homoserine or Lmethionine plus L- threonine with a mutant strain of *Corynebacterium glutamicum*. In the wild type of this organism concerted feed back inhibition is by both lysine and threonine. Inhibition does not occur when only one is present. In this particular mutant absence of biosynthetic homoserine derived from aspartic acid causes lysine to accumulate



Brevibacterium flavum. In this organism the L- lysine pathway is regulated at aspartate kinase which is the only enzyme sensitive to feed back inhabitation by lysine. Mutants resistant to lysine analogues therefore over produce the amino acid

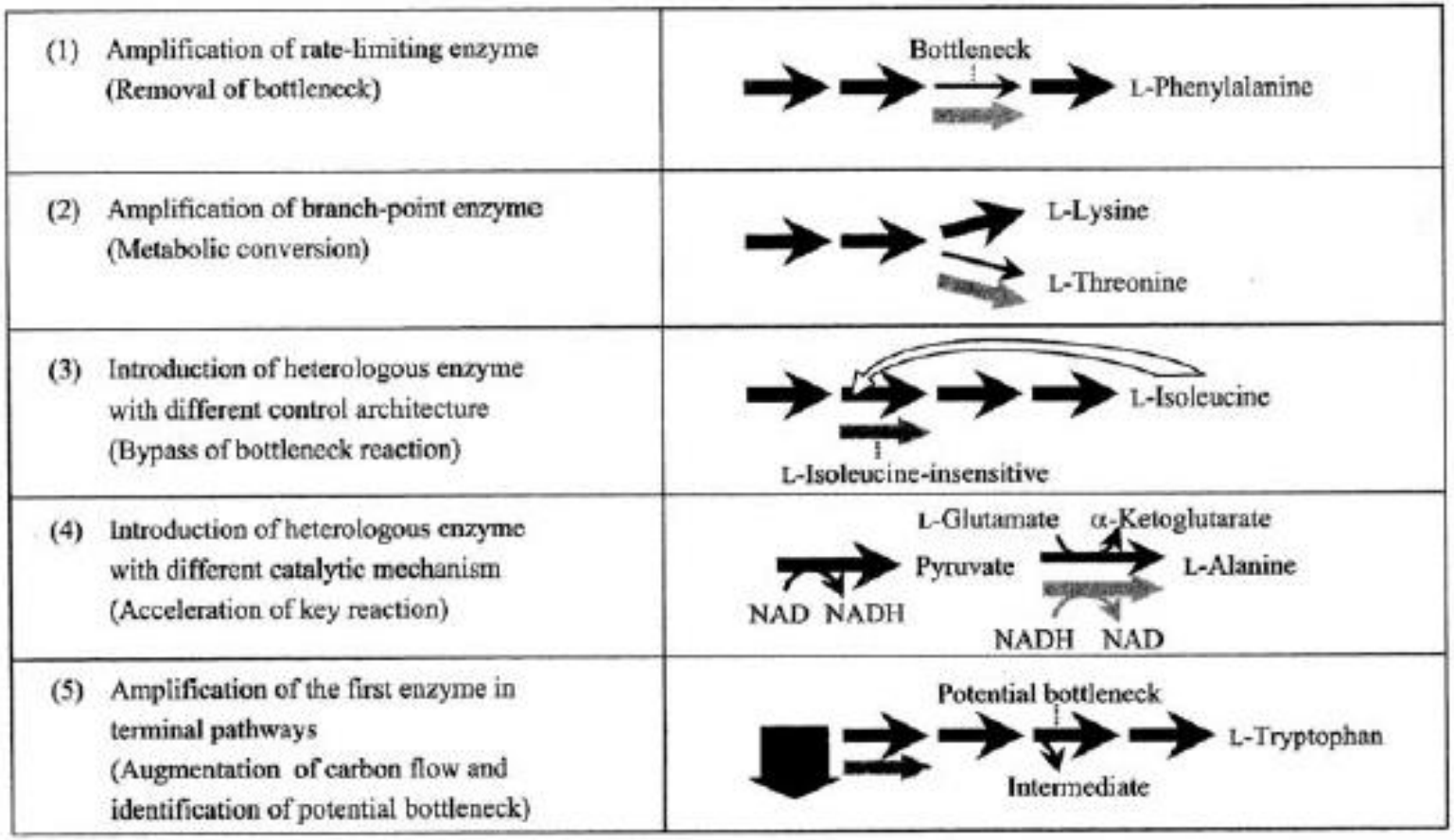
Table 21.5 Improvements in amino acid production through the cloning of different genes

Amino acid produced	*Microorganisms*	*Gene donor*	*Cloned gene or enzyme*	*Yield mg/mL*
L-alanine	*E. coli*	*B. stearothermophilus*	Ala dehydrogenase	
D-alanine	*E. coli*	*Ochrobactrum anthropi*	D-aminopeptidase	200
L-histidine	*C. glutamicum*	*C. glutamicum*	His G, D, C, B	15
	S. marcescens	*S. marcescens*	His G, D, B	43
L-isoleucine	*C. glutamicum*	*C. glutamicum*	Hom dehydrogenase	11
	B. flavum	*E. coli*	ilv A	21
L-lysine	*C. glutamicum*	*C. glutamicum*	Lys A, dap A, B, D, Y	
	C. glutamicum	*E. coli*	Asp A	
L-phenylalanine	*C. glutamicum*	*C. glutamicum*	aro F, chorismate mutase, PRDH	28
	C. glutamicum	*E. coli*	aro G, Phe A	
	B. lactofermentum	*B. lactofermentum*	aro F, E, L, PRDH	21
L-proline	*S. marcescens*	*S. marcescens*	Pro A, B	75
L-threonine	*E. coli*	*E. coli*	Thr A, B, C	55
	C. glutamicum	*C. glutamicum*	hom dehydrogenase, hom kinase, Thr C	51
	B. lactofermentum	*B. lactofermentum*	ppc, hom dehydrogenase, hom kinase	33
	B. flavum	*E. coli*	Thr B, C	27
	S. marcescens	*E. coli*	ppc	60
L-tryptophan	*E. coli*	*E. coli*	Trp A, E, R, tna A	40
	C. glutamicum	*C. glutamicum*	Trp E, aro F, chorismate mutase, PRDH	45
L-tyrosine	*C. glutamicum*	*E. coli*	Aro F	9

(i) the terminal pathways of the amino acid synthesis
(ii) the central metabolic pathway for producing the amino acid
(iii) the transport process for secreting amino acid

Metabolic Engineering to Improve Transport of Amino Acids Outside the Cell

The aim of strain improvement is to prevent feedback inhibition when the amino acid accumulates intracellularly. One manner in which feedback inhibition can be avoided is through increased efflux of the amino acid. A gene which codes for increased efflux has been introduced into *E.c*oli resulting in a vastly increased production of L-cysteine



Strategies to Modify the Terminal Pathways

The strategies for modifying the terminal pathways are indicated in Fig.

1. *Amplification of rate limiting enzyme*: The gene coding for the rate limting enzyme in the biosynthetic pathway is amplified. Large increases have been observed when this technique was applied to L-phenyl alanine production in *Corynebacterium glutamicum*.

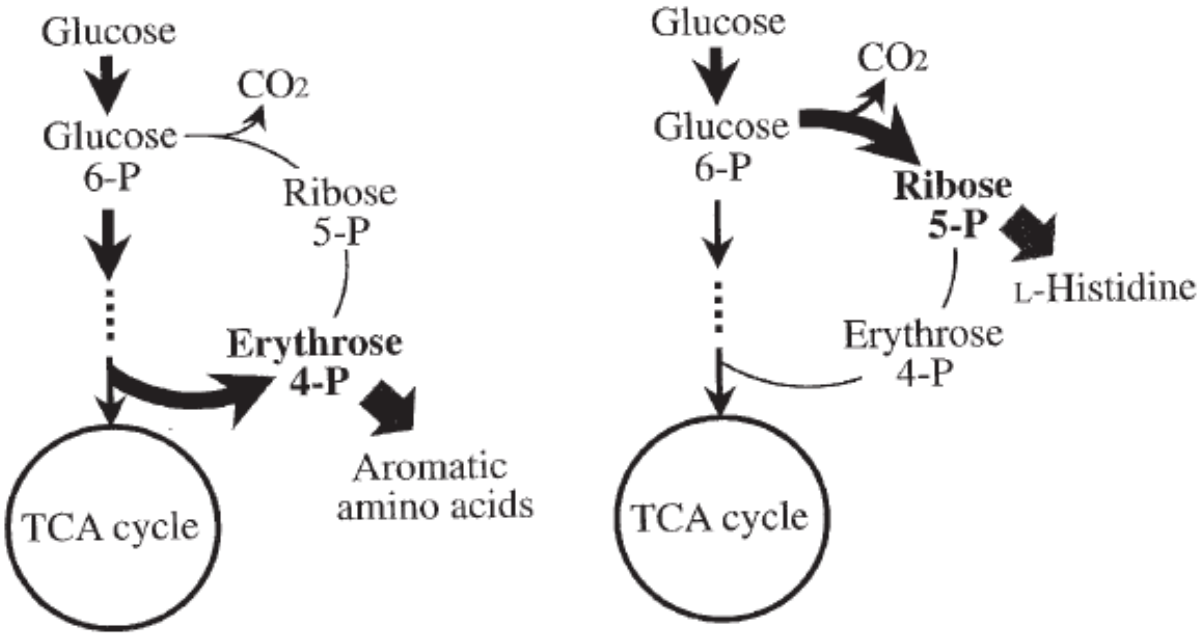
2.

***2. Amplification of branch-point enzyme*:** The gene coding for the branch-point enzyme is amplified to redirect the common intermediate to another amino acid. It has been used successfully in converting L-lysine to L-tryptophane and L-tyrosine to Lphenylalanine.

3. *Introduction of a different enzyme* *able to produce the same end amino acid*: The gene for a different enzyme for the same end amino acid is introduced. The enzyme creating the bottle neck is thus bypassed. This has been used for increased L-isoleucine production in *Corynebacterium glutamicum*.

4. *Introduction of a more functional enzyme* *than the native one*: Introduction of an enzyme which is more active than the native one thereby enhancing the production of the amino acid. This has enhanced the production of L-alanine production by *Corynebacterium glutamicum* when L-alanine dehydrogenase from *Arthrobacter oxydans* was engineered into it .

5. *Amplification of the first enzyme in the terminal pathway*: The first enzyme in a pathway diverging from central metabolism is amplified to increase the flow in that pathway; any bottleneck is removed by the increased down the pathway. This strategy has been applied to obtain increased yield of L tryptophan by *Corynebacterium glutamicum.*



Strategies for Increasing Precursor Availability

The fermentation is usually batch or fed-batch. In batch cultivation all the nutrients are added at once at the beginning of the fermentation, except for ammonia which is added intermittently to help adjust the pH, and fermentation continues until sugar is exhausted. In a fed-batch process, the fermentor is only partially filled with medium and additional nutrients added either intermittently or continuously until an optimum yield is obtained. The fed-batch appears preferable for the following reasons:

(a) **Most amino acid** production requires high sugar concentrations of up to 10%. If all were added immediately, acid would be quickly produced which will inhibit the growth of the microorganisms and hence reduce yield.

(b) **Where auxotrophic mutants** are used, excess supply of nutrients leads to reduced production due to overgrowth of cells or feed back regulation by the nutrient.

(c) **During the lag phase of growth**, the oxgen demand of the organism may exceed that of the organism leading to reduced growth.

Raw Materials

The main raw materials used are cane or beet molasses and starch hydrolysates from corn or cassava as glucose. In the US, the preferred carbon source is corn syrup from corn, whereas in Europe and South America it is beet molasses.

As nitrogen source, inorganic sources such as ammonia or ammonium sulfate is generally used. Phosphates, vitamins and other necessary supplements are usually provided with corn steep liquour.